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Acta Physiologica Scandinavica

Vol. 43 Supplementum 148

OF MICHIGAN
OCT 1958
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BIRGER BLOMBÄCK

STOCKHOLM 1958

UNIVERSITY OF MICHIGAN
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Acta Physiologica Scandinavica

Vol. 43 Supplementum 148

FROM CHEMISTRY DEPARTMENT II
(*Head:* PROFESSOR ERIK JORPES),
KAROLINSKA INSTITUTET, STOCKHOLM, SWEDEN

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KATALOG OCH TIDSKRIFTSTRYCK
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This dissertation is a discussion and summary of the following seven papers:

I. BLOMBÄCK, B. and BLOMBÄCK, M., Purification of human and bovine fibrinogen, Arkiv för kemi 1956. *10*. 415.

II. BLOMBÄCK, B., On the properties of fibrinogen and fibrin, Arkiv för kemi 1958. *12*. 99.

III. BLOMBÄCK, B. and LAURENT, T. C., N-terminal and light-scattering studies on fibrinogen and its transformation to fibrin, Arkiv för kemi 1958. *12*. 137.

IV. BLOMBÄCK, B. and YAMASHINA, I., On the N-terminal amino acids in fibrinogen and fibrin, Arkiv för kemi 1958. *12*. 299.

V. BLOMBÄCK, B. and VESTERMARK, A., Isolation of fibrinopeptides by chromatography, Arkiv för kemi 1958. *12*. 173.

VI. BLOMBÄCK, B., Studies on the action of thrombic enzymes on bovine fibrinogen as measured by N-terminal analysis, Arkiv för kemi 1958. *12*. 321.

VII. BLOMBÄCK, B., BLOMBÄCK, M. and NILSSON, I. M., Coagulation studies on "Reptilase," an extract of the venom from Bothrops jararaca, Thromb. Diath. haem. 1957. *1*. 76.

One of the most important contributions to our present knowledge of fibrinogen and fibrin was given by the work of Olof Hammarsten. He frequently stressed the importance of purification of the individual substances participating in blood coagulation. Speaking of the experiments which had been made on plasma and blood in order to elucidate the nature of fibrin formation, Hammarsten said in a lecture given in 1876:

"Och ehuruväl jag visst icke vill neka, att med ett sådant material rätt mången vigtig iakttagelse kan göras, är det dock fortfarande min bestämda öfvertygelse, att man ensamt på den vägen ej kan utreda frågan. Vid sådana undersökningar rör man sig med alldeles för många obekanta, och min öfvertygelse har derför alltid varit, att man här, lika som öfverallt inom kemien, måste i första hand söka isolera och i möjligast rent tillstånd framställa de i den kemiska reaktionen intresserade ämnena."[1]

[1] Although I certainly do not wish to deny that fairly many important observations can be made with the use of such material, I am still fully convinced that the problem cannot be elucidated in this way alone. In such investigations, one is dealing with far too many unknown factors; consequently, it has always been my conviction that here, as in every other field of chemistry, one must in the first place try to isolate and to produce in the purest possible state the substances involved in the chemical reaction.

Historical Outline

"Recite thou, in the name of thy Lord who created; —
Created man from CLOTS OF BLOOD." (The Koran)

Coagulation of the blood and its red colour are such evident phenomena that their observation can be traced back to the earliest historical times. It was not, however, until the end of the 17th century that the structural basis of the blood clot, fibrin, was described by Malpighi as a white fibrous substance obtainable from the red blood clot through washing with water.

While it had formerly been widely believed that coagulation occurred through some kind of aggregation of the red blood cells, Hewson showed in about 1770 that the coagulation process is independent of the formed elements, and takes place in the blood lymph (plasma). Like Malpighi, Hewson observed that neutral salts can retard or completely inhibit coagulation of the blood. He found that when the blood cells are allowed to settle in salted blood, the clear lymph can be decanted off and stored. On dilution with water, the stored lymph coagulates spontaneously. Salting of blood was the first principle which made possible a storage of unclotted blood and plasma. It was also the sole principle until decalcification with oxalate and citrate was introduced at the end of the last century. Hewson expressed the view that, prior to coagulation, the fibrin is present as a solute in the plasma. Half a century later, Johannes Müller in Bonn (1832) was able to confirm this opinion, when he filtered off the blood cells from frog's blood before coagulation occurred.

Berzelius, and later Nasse (1836), made thorough analyses of blood in order to determine its content of fibrin, or rather its precursor, which Virchow (1847) named fibrinogen. Independently, this name was later used by Denis de Commercy (1859) as well, who had until then used the expression "plasmine". He wrote:

"Je crois devoir répéter encore que je n'entends pas dénommer ainsi la fibrine liquide, la fibrine plasmique, mais bien la matière nullement fibrineuse qui est l'origine de cette fibrine, la lymphe coagulable, la substance que j'appelais séro-fibrine, et qu'on pourrait aussi désigner sous le nom de fibrinogène, si un pareil mot pouvait être admis. Elle est liquide dans le sang, et, dès qu'il est tiré de ses vaisseaux, elle ne tarde pas à se dénaturer."

Denis showed that fibrinogen can be salted out from plasma, kept incoagulable by means of sodium sulphate, on saturation with sodium chloride. He observed that the fibrinogen precipitated with sodium chloride can be dissolved completely on dilution with water, but that some 10 minutes later it is spontaneously transformed into fibrin, which then gradually redissolves. Denis was also among the first to suggest the possibility of precipitating globulins by dilution of the plasma with water. The importance of Denis' work is perhaps best expressed in the words of Hammarsten, 20 years later:

> It is true that we now know more about this (fibrin formation) than Denis did, but what he believed he knew is nevertheless in better agreement with the true conditions than any other theory. This is why I have also been able to state that Denis was closer to the truth than his predecessors and his immediate successors.

Denis' work was carried out at about the same time as Alexander Schmidt's studies on thrombin formation. Thus, Denis' *Mémoire sur le sang* appeared in 1859, two years before Alexander Schmidt presented his coagulation theory, which still forms the basis of our modern concepts of coagulation.

Hammarsten, like Buchanan before him and Arthus and Pagés after him, had been struck by the similarity between the curdling of milk and the coagulation of blood. It was presumably this likeness which made him take up the study of fibrinogen and of fibrin formation, after completing his investigations on casein. In 1876—1880, Hammarsten published his studies on fibrinogen. He purified fibrinogen from horse plasma through repeated precipitations with sodium chloride at half-saturation. He stated that solutions of pure[1] fibrinogen do not coagulate spontaneously, but rapidly on addition of thrombic enzyme; the coagulability, however, sometimes more or less rapidly diminished on storage in solution. Hammarsten also found that the solubility of fibrinogen is dependent on the presence of salts, and that exposure to a temperature of + 52 to + 55° C is sufficient to cause irreversible denaturation (Hammarsten 1876). Denaturation of plasma fibrinogen at + 56 to + 58° C was independently described by Frédericq (1877).

Hammarsten's method formed the basis of the standard procedures for preparation of fibrinogen. Modifications of his method have been made by Rettger (1909), Howell (1910), Bordet (1920), Florkin (1930), Eagle (1935) and Chargaff & Bendich (1943).

[1] Some of Hammarsten's fibrinogen preparations seem to have been more than 90 % coagulable with thrombin.

Rettger increased the stability of the fibrinogen solutions by treating them with barium phosphate, which adsorbs prothrombin. Bordet (1920) used tricalcium phosphate, the prothrombin (serozyme) adsorbing capacity of which had been described by Bordet & Delange (1914). Bordet and his co-workers had previously observed that prothrombin can be adsorbed not only to calcium phosphate, but also to calcium fluoride (Bordet & Gengou 1904), and to barium sulphate (Bordet & Delange 1912). Florkin, who studied the solubility of fibrinogen in different salt solutions, carried out all procedures at low temperature in order to minimize the risks of denaturation.

In 1909, Mellanby described a method of preparing bird fibrinogen, in which he precipitated the fibrinogen together with other globulins by dilution of plasma with water at a slightly acidic reaction, a principle which, as stated by Mellanby, had already been used by Alexander Schmidt.

The precipitability of globulins and albumins with ammonium sulphate, as well as with other salts, had been studied by Hofmeister and his school as long ago as at the end of the 19th century. Ever since that time, ammonium sulphate has been widely used in protein chemistry. McLean (1920), Smith, Warner & Brinkhous (1934), Milstone (1941) and Astrup & Darling (1942) precipitated the fibrinogen from plasma at 25 to 30 % saturation with ammonium sulphate. Before precipitation, Smith *et al.* treated the plasma with $Mg(OH)_2$ in order to remove the prothrombin. Milstone found that the fibrinogen preparations were usually contaminated by a lytic factor which could, however, be removed by precipitating the fibrinogen with ammonium sulphate ten times. Astrup & Darling claimed that ammonium sulphate precipitation of the fibrinogen in plasma treated with tricalcium phosphate gave a product superior to that of Hammarsten and of Mellanby, with respect to both purity and stability.

Although neutral salts have usually been employed for precipitation and purification of fibrinogen, buffer salts have also been used. Thus Jaques (1943) utilized its low solubility in concentrated phosphate buffers, which had previously been studied by Florkin (1930). Avery & Munro (1948) considered that this technique yields a product which is superior to that obtained by purification with either ammonium sulphate or sodium chloride.

Fibrinogen differs from most of the other plasma proteins by its low solubility at low temperatures. Freezing out the fibrinogen from plasma thus provides a simple way of obtaining a preparation of high purity (Ware, Guest & Seegers 1947). The plasma,

frozen down to — 30° C, is allowed to thaw gradually. The fibrinogen, which remains undissolved, is washed with chilled isotonic saline and then dissolved by increasing the temperature.

Crystallization of fibrinogen has been reported by Laki (1942) and Bagdy (1949). The former gave, however, no definite data about the coagulability of the product. Bagdy's crystalline preparation was coagulable to 85—96 %. Bagdy used ammonium sulphate fractionation, followed by isoelectric precipitation at low temperature. Crystallization took place from a weakly ammoniacal solution at low ionic strength, when the pH fell on standing. The stability in solution was less satisfactory, because the preparations often contained an active fibrinolytic enzyme. In a later publication by Szára & Bagdy (1953) a modified technique was described which was stated to result in increased purity of the preparation, the coagulability being 95—98 %. This fibrinogen was not reported to be crystalline.

At the end of the 1930's and the beginning of the 1940's, the Harvard group, under the direction of Cohn, carried out careful physico-chemical investigations of the behaviour of proteins in ethanol-water solutions. During World War II, this resulted in elaboration of the well-known 5-variable system for fractionation of plasma proteins. In Cohn's method 6 (1946) about 80 % of the fibrinogen in plasma was obtained in fraction I, some 60 % of which was reported to consist of fibrinogen according to the electrophoretic analysis. This fraction also contains small quantities of prothrombin and is therefore not stable in solution. Later improvements in the technique, such as collection of the blood over cation exchangers and in siliconized vessels, as well as adsorption of prothrombin on barium sulphate, have increased the stability of fibrinogen in fraction I (Surgenor, Alexander, Goldstein & Schmid 1951, Edsall & Dandliker 1951).

Further purification of fraction I by ethanol fractionation has been described by Morrison, Edsall & Miller (1948). With this procedure, fibrinogen is first precipitated at pH 5.1 at an ethanol concentration of 2 vol. %, most of the contaminants remaining in solution. The fibrinogen fraction is then dissolved in a citrate buffer at pH 6.3, after which the globulins insoluble in the cold are removed at 0° C and an ethanol concentration of 0.5 to 1 vol. %. The protein in the final product is described as being between 93 och 98 % coagulable with thrombin. However, on electrophoresis and ultracentrifugation, not even the purest preparations have been found to be homogeneous. On ultracentrifugation, the main component had the S_{20} value of 8.5 S. A component with

$S_{20} = 15$ S was also present, and sometimes a more rapidly sedimenting component as well (Edsall 1953).

The conditions under which fibrinogen precipitates from plasma are not the same for all species. Consequently, the aforementioned purification procedure, elaborated by Morrison *et al.* for human plasma, is not strictly applicable to bovine plasma. A modified procedure suitable for bovine plasma was therefore worked out by Morrison, Shulman & Blatt (1951), the final product being coagulable to 90 %. Laki (1951) obtained, however, by ammonium sulphate fractionation of bovine fraction I, a stable fibrinogen preparation which was coagulable with thrombin to 95 % and which has been extensively used for the physico-chemical characterization of fibrinogen.

In England, diethyl ether was used instead of ethanol for fractionation of human plasma proteins (Kekwick, Mackay & Record 1946, Kekwick & Mackay 1954). In the method for purification of fibrinogen elaborated by Kekwick, Mackay, Nance & Record (1955) the fibrinogen is precipitated from plasma by adding 11 vol. % ether. Contaminants are extracted from this fraction by 8 vol. % ether-citrate-saline, after which the fibrinogen is extracted by a citrate buffer, ionic strength 0.3, leaving cold-insoluble globulins as a residue. A fraction rich in plasminogen is subsequently removed by precipitation at low ionic strength and 0.5 vol. % ether. The fibrinogen precipitated from the supernatant of this fraction is a highly purified, stable fibrinogen with a coagulability exceeding 97 %. All procedures are carried out in the cold. This fibrinogen showed only one component in the ultracentrifuge, and on electrophoresis at pH 8.0. It is free from prothrombin and plasmin and, practically speaking, free from plasminogen or its proactivator. Immunological studies, however, indicate traces of contamination with some plasma proteins (Caspary 1956).

Properties of Fibrinogen and Fibrin

1. *Physico-chemical properties.* — During the past seventy years, the physico-chemical properties of human and bovine fibrinogen and fibrin preparations have been extensively studied. The low solubility of fibrinogen in a weakly acidic watery solution or in concentrated salt solutions was shown, as mentioned above, already by Denis and Hammarsten. The latter also pointed out that the solubility was appreciably increased in an alkaline

watery solution. Hammarsten was thus able to obtain fibrinogen solutions at very low ionic strength, by dialyzing against 0.006—0.003 % sodium hydroxide. When these solutions were diluted, the fibrinogen precipitated. The euglobulin nature of fibrinogen has later been repeatedly confirmed.

The isoelectric point of fibrinogen of varying purity has been the subject of several investigations.

In 1927 Norbō made a careful study of the hydrochloric acid-binding capacity of human and horse fibrinogen, prepared by sodium chloride fractionation. From the electrometric titration curve, he obtained an isoelectric point of about 5.5 for fibrinogen in 0.85 per cent sodium chloride solution. The maximal acid-binding capacity amounted to 0.06 mg of hydrochloric acid per mg of fibrinogen.

The electrophoretic properties of human fibrinogen were studied by Stenhagen in 1938, using the Tiselius apparatus. From the mobilities at different pH values he calculated an isoelectric point between 5.2 and 5.6. By means of electrophoresis, Seegers, Nieft & Vandenbelt (1945) estimated the isoelectric point of bovine fibrinogen to be about 5.5.

Measurements of the Z-potential of human and bovine fibrinogen at different pH values gave a zero value at pH 5.8 (Sheppard & Wright 1954, Abe, Sheppard & Wright 1955). This zero potential point was considered to be the isoelectric point of these proteins.

A small change in the net charge of fibrinogen seems to occur during its transformation to fibrin, since the isoelectric point of fibrinogen and fibrin in urea solution is 5.5 and 5.6, respectively (Mihalyi 1950).

Measurements of X-ray diffraction, osmotic pressure, intrinsic viscosity, sedimentation and diffusion constants, flow-birefringence and light scattering have been made by several authors.

The X-ray diffraction pattern of fibrinogen and fibrin indicates that they belong to the keratin-myosin group of proteins (Bailey, Astbury & Rudall 1943). In both proteins, an α to β transformation seems to take place at high tension.

Fibrinogen has a low osmotic pressure and the pressure is not much dependent on pH between 5.9 and 9.2 (Nanninga 1946).

An intrinsic viscosity of 0.25 has been reported for human and bovine fibrinogen by Oncley, Scatchard & Brown (1947), Hocking, Laskowski & Scheraga (1952) and by Caspary & Kekwick (1957).

Measurements of the sedimentation constant (S_{20}) of human fibrinogen were first made by Holmberg (1944). He found a value of 8.5, which is in agreement with the figure of Oncley, Scatchard

& Brown. Koenig & Pedersen (1950) also obtained a sedimentation constant of 8.4 to 8.6 for bovine fibrinogen. Caspary & Kekwick (1954, 1957), however, for a purer human fibrinogen, found a somewhat lower sedimentation constant, i. e., 7.6. The diffusion constant reported for this purified fibrinogen is higher than that of Holmberg (1944).

By means of osmotic pressure measurements, Nanninga (1946) found a molecular weight of 441,000 for bovine fibrinogen. Oncley *et al.* (1947) arrived at the figure 400,000 for human fibrinogen, after analysis of viscosity and sedimentation data. Holmberg (1944), on the basis of sedimentation and diffusion data, calculated a molecular weight of 700,000 for human fibrinogen. Using the same technique of analysis, Caspary & Kekwick (1954, 1957) found a molecular weight of only 341,000 for a highly purified human fibrinogen coagulable to 97—98 %, and Shulman (1953) found 330,000 for a bovine fibrinogen. The sedimentation and diffusion data of Caspary & Kekwick suggest a disaggregation of the 340,000 unit, at low protein concentration, into several subunits.

The molecular properties have also been investigated by light-scattering measurements. Steiner & Laki (1951) calculated a molecular weight of 540,000 for bovine fibrinogen, whereas Katz, Gutfreund, Shulman & Ferry (1952) assumed 340,000 to be the most probable molecular weight. Using the same method, Hocking, Laskowski & Scheraga (1952) obtained the figure 407,000.

The high intrinsic viscosity of fibrinogen indicates a somewhat asymmetric molecule. From flow-birefringence measurements and related data of human fibrinogen, Edsall, Foster & Scheinberg (1947) described the most probable molecular shape as a prolate ellipsoid 700 Å long, with an axial ratio of 18 to 1. Such a model is also applicable to bovine fibrinogen, as indicated by flow-birefringence and viscosity measurements (Hocking, Laskowski & Scheraga 1952), and by light-scattering studies (Katz, Gutfreund, Shulman & Ferry 1952). A somewhat longer molecule (about 800—840 Å) was calculated from the light-scattering data of Steiner & Laki (1951) and Hocking, Laskowski & Scheraga (1952).

Besides differences in the theoretical interpretation of the analytical data, one important cause of the variable results obtained by the different authors seems to lie in the varying techniques used in the preparation of fibrinogen. The results may have been influenced by impurities in the final product, as well as by molecular alteration in the course of preparation. In reviewing the

situation in 1954, Edsall considered the most probable molecular weight to be between 325,000 and 340,000, and expressed the view that, at least as a temporary working model, the prolate ellipsoid 600—700 Å long and with an axial ratio in the order of 18—20 was useful. The electron-microscopic studies of Hall (1949) and Siegel, Mernan & Scheraga (1953) are also in conformity with this view with respect to size and shape of the fibrinogen molecule.

2. *Chemical properties.* — The nitrogen content is higher in fibrinogen than in albumin and gamma globulin (*cf.* Tristram 1953). In horse fibrinogen and fibrin, Hammarsten (1880) found 16.66 % and 16.91 % nitrogen, respectively. In human fibrinogen, the nitrogen content amounts to 17.1 % (Bailey 1944) and in human fibrin to 16.9 % (Brand, Kassell & Saidel 1944). The amide nitrogen in human fibrinogen is 1.49 % (*cf.* Tristram 1953).

The amino acid composition has some characteristic features compared with albumin, e. g. the tryptophan, serine, threonine, methionine, arginine and aspartic acid content is higher (*cf.* Tristram 1953). The amino acid distribution shows many similarities to myosin and keratin (Bailey 1944).

The total sulphur was found by Hammarsten to be 1.25 % in fibrinogen and 1.10 % in fibrin.

Carbohydrate components of fibrinogen and fibrin. — Fibrinogen and fibrin contain several carbohydrate components, in similarity to a number of other plasma proteins (*cf.* Schultze 1955, Jorpes & Yamashina 1956).

Seibert, Pfaff & Seibert (1948) found the carbohydrate content to be 1.2 % of the dry weight in Cohn's fraction I from human plasma, a more purified fraction having a content of 1.0 %. The carbazole reaction was used in these estimations.

Using the Schaffer-Somogyi method for determination of reducing sugar, Consden (1953) found fibrinogen to contain 4.6 % reducing sugar and 1.1 % hexosamines (Elson-Morgan method). This fibrinogen was prepared with Kekwick's ether method. In human fibrin, little if any change in carbohydrate composition was found. In both fibrinogen and fibrin, galactose, mannose and glucosamine were identified by means of paper electrophoresis and chromatography. Preliminary ionophoresis indicated the absence of uronic acid in fibrin (Consden & Stanier 1952).

Fibrinogen and fibrin from different species were analyzed for their carbohydrate components by Szára & Bagdy (1953). They found from 1.6 to 2.0 % hexoses in different fibrinogens, the figure for fibrin being about 16 to 19 % lower than that for the corresponding fibrinogen. The amount of hexosamine was between 0.5

and 0.6 % in both fibrinogen and fibrin. Galactose, mannose and glucosamine could be identified in the two proteins by paper chromatography.

The difference between the hexose content of fibrinogen and fibrin was regarded to indicate splitting off of a polysaccharide during coagulation (Bagdy & Szára 1955). These workers found that the splitting off of hexoses starts before gel formation begins, and reaches its maximum before coagulation is complete. They suggested that the splitting off of the polysaccharide was a phenomenon associated with the proteolytic activity of thrombin.

The presence of sialic acid[1] in serum proteins has been demonstrated by several workers (Odin & Werner 1952, Werner & Odin 1952, Böhm, Dauber & Baumeister 1954, Böhm & Baumeister 1955, Odin 1955, Yamashina 1956).

It is evident from the investigations of Böhm & Baumeister (1955) that human fibrin obtained after coagulation of plasma contains small quantities of sialic acid. The substance was isolated as methoxy neuraminic acid. Determined with Bial's reagent, the fibrin from healthy subjects contained 0.64 to 0.89 per cent of "neuraminic acid."

N-terminal amino acids. — N-terminal analysis of bovine fibrinogen was first made by Bailey (1951), using the fluorodinitrobenzene (FDNB) method of Sanger. One mole of tyrosine was found in 200,000 g and one mole of glutamic acid in 300,000 g of protein. Analysis of bovine fibrinogen and fibrin in the same year (Bailey, Bettelheim, Lorand & Middlebrook 1951) showed a difference between the N-terminal amino acids of bovine fibrinogen and fibrin. Thus, the glutamyl residues in bovine fibrinogen were replaced in fibrin by glycyl residues. This indicated a splitting off of peptide material during the transformation. Thorough analyses of the N-terminal amino acids in bovine fibrinogen and fibrin have been made since 1951, using Sanger's technique (Bettelheim & Bailey 1952, Lorand & Middlebrook 1952, Bailey & Bettelheim 1955). Some uncertainty seems to exist regarding the number of N-terminal amino acids per unit weight of fibrinogen and fibrin, due to difficulties in the quantitative analyses of N-terminal amino acids by the FDNB method. Lorand & Middlebrook (1952) found, after correction for destruction, one N-terminal glutamyl and two N-terminal tyrosyl residues in a 450,000 unit weight of bovine fibrinogen. In the corresponding

[1] Sialic acid has been proposed as the group name for acylated neuraminic acids (Blix, Gottschalk & Klenk 1957).

amount of fibrin they found two tyrosyl and four glycyl N-terminal residues. Bailey & Bettelheim (1955), assuming a molecular weight of 330,000 for bovine fibrinogen, considered the most probable number to be one glutamyl and two tyrosyl end-group residues per mole of fibrinogen, and two tyrosyl and two or three glycyl residues in an approximately equal weight of fibrin.

Species differences of fibrinogen were revealed by end-group studies, when Lorand & Middlebrook (1953) showed that human fibrinogen contained alanyl residues, instead of the glutamyl residues present in bovine fibrinogen. They found one N-terminal tyrosyl residue in 2.2×10^5 g of protein and one alanyl residue in 3×10^5 g.

Bailey & Rosemeyer (cited by Sanger 1955) reported tyrosyl and threonyl residues in horse fibrinogen, but no quantitative evaluation was given.

Different types of clots have been the subject of end-group studies by Middlebrook (1955). The results of Lorand & Middlebrook (1952) were confirmed with respect to clots obtained from fibrinogen after addition of thrombin, or by recalcification from plasma. However, when the plasma clots were treated by washing with urea solution or dialysis against monochloroacetic acid, one glycyl chain per 450,000 unit weight of protein was removed.

3. *Solubility of fibrin.* — It is now known that fibrin can be prepared in two forms, one of which contains much stronger bonds than the other. This conclusion is based on the difference in solubility which characterizes these fibrins.

The investigations of Robbins (1944) indicated that fibrin can be classified into two types, according to its solubility in dilute alkalis and acids. The fibrin obtained by Robbins from purified fibrinogen, after addition of thrombin, could be dissolved in these solvents, but not the fibrin formed by thrombin in the presence of ionized calcium and a serum factor.

Opinions had earlier been at variance regarding the solubility of fibrin in urea. Laki and Lorand (Laki & Lorand 1948, Lorand 1948) clearly demonstrated that two types of fibrin could be distinguished also with respect to their solubility in urea. One, insoluble in 30 % urea, was formed in the presence of calcium and a heat-labile serum factor. The other, with greater solubility, was prepared from pure fibrinogen in the presence of thrombin alone. Lorand (1948, 1950) found the viscosity of fibrin dissolved in urea to be the same as that of fibrinogen in this solvent, thus indicating depolymerization of fibrin in urea solution. On removal of the urea by dialysis, the fibrin once more polymerized and precipi-

tated. It was also observed in X-ray studies that fibres of the insoluble fibrin were more readily converted from the α to the β configuration by tension. Lorand suggested that the serum factor necessary for formation of the insoluble fibrin might act as a cement between the fibrin units.

Fibrin exhibits the same properties of solubility as in urea in a number of salt solutions, such as lithium chloride (3 *M*), lithium bromide (2 *M*), sodium bromide (2 *M*) and sodium iodide (1 *M*): Shulman & Katz (1952).

Even purified fibrinogen is often found to be contaminated with the serum factor (Loewy & Edsall 1954). Shulman (1953) showed that the serum factor could be removed from fibrinogen by several reagents (urea, sodium bromide) and that it could be restored by addition of serum albumin (fraction V). Human albumin prepared by the ether fractionating technique was, however, found to have no such activity (Lorand 1954).

The studies of Loewy & Edsall (1954) showed that the urea-soluble clot (fibrin *s*) could be converted into the urea-insoluble clot (fibrin *i*) in the presence of the serum factor and calcium. Reagents reacting specifically with sulfhydryl groups, *e. g.* mercurials, strongly inhibited the formation of fibrin *i*. An interesting finding was that sulfhydryl compounds could replace the serum factor in the formation of fibrin *i*. On the basis of these findings, Loewy & Edsall suggested that the structure of fibrin *i* involves the formation of new intermolecular disulphide cross-links within the fibrin clot.

The "fibrin-stabilizing factor" from human plasma has been purified by Kekwick and Lorand (cited by Lorand 1954). About 75 % of the activity was found in a small fraction, rich in prothrombin, containing about 1—2 % of the total plasma proteins. Further purification of this factor has recently been reported by Lorand & Jacobsen (1958).

Transformation of Fibrinogen to Fibrin

In the following, I shall deal chiefly with the proteolytic course of this reaction. The relation between this proteolysis and the subsequent polymerization is not yet understood. As far as the physico-chemical studies of fibrin polymerization are concerned, I shall confine myself to an account of those relevant to the present investigations. A review of this enormous field of research has recently been given by Scheraga & Laskowski (1957).

According to the coagulation theory first presented by Alexander Schmidt in 1861, coagulation proceeds in two phases. In the first phase, an active enzyme is formed from an inactive precursor by means of activating zymoplastic substances. In the second phase, the ferment causes a conversion of fibrinogen to fibrin. This theory is still valid.

In a paper on fibrin formation published in 1872, Schmidt established, with excellent precision, the enzymatic nature of thrombin. He wrote as follows:

"Die Gründe, welche mich veranlassen, diesen Körper für ein Ferment anzusprechen, sind folgende:

1. Beliebig kleine Mengen desselben bewirken in einer Flüssigkeit mit gegebenem Gehalt an Fibringeneratoren eine ebenso erschöpfende Fibrinausscheidung wie beliebig grosse, so dass nach Beendigung des Processes weiterer Zusatz dieses Körpers keine Fibrinausscheidung mehr bewirkt und das Gewicht des erhaltenen Faserstoffes stets das gleiche ist. Es entsteht also immer so viel Faserstoff, als bei dem gegebenen Gehalt an Fibringeneratoren überhaupt entstehen konnte, aber die Gerinnungszeiten sind von dem Gehalt der Flüssigkeit an diesem dritten Körper abhängig, so dass man es, indem man diesen Gehalt variirt, in seiner Hand hat, den Ablauf des Processes auf Secunden zusammenzudrängen resp. ihn über Tage auszudehnen. Ein chemischer Verbrauch dieser Substanz findet also offenbar nicht Statt.

2. Die Wirkung des in Rede stehenden Körpers steigt mit der Temperatur bis zu einer Grenze, welche mit der Temperatur des Thierleibes übereinstimmt; über diese Grenze hinaus wirkt die Temperatur nachtheilig. Siedhitze zerstört seine Wirksamkeit, bei annähernd 0° erhält er sich unbegrenzt lange, seine Wirkung wird aber durch diese Temperatur gehemmt.

3. Ist die Faserstoffgerinnung beendet, so kann man mit der nach Entfernung des Faserstoffes zurückbleibenden Flüssigkeit in anderen, die beiden Fibringeneratoren enthaltenden, Flüssigkeiten von Neuem die Gerinnung bewirken, und so fort. Aber man beobachtet bei solchen Uebertragungen doch eine allmählig zunehmende Schwächung der Wirkung, welche vielleicht damit zusammenhängt, dass neben dem Faserstoff möglicherweise noch andre Fermentationsprodukte entstehen, welche für die Fermentation selbst ein bei den Uebertragungen wachsendes Hinderniss abgeben.

Ich glaube, dass diese Gründe mich berechtigen, von einem Fibrinferment zu sprechen. Welcher Art aber der Fermentationsprocess selbst ist, welchen Spaltungen resp. Verbindungen die beiden Fibringeneratoren bei der Fibrinbildung unterliegen, darüber wage ich keinerlei bestimmte Aussage zu machen..."

It is apparent from these statements that the concept of splitting of the fibrinogen during its conversion to fibrin was not foreign to Alexander Schmidt.

Hammarsten, like Schmidt, showed the transformation to fibrin to be an enzymatic process. In contrast to Schmidt, Hammarsten held the view that only fibrinogen and thrombin were necessary for the formation of fibrin. Schmidt was of the opinion that fibrin

was formed from two protein components of the plasma: fibrinogen and paraglobulin.

Hammarsten (1876) also pointed out the importance of the salt concentration for fibrin formation. The action of the ferment is hastened by salt in low concentrations, whereas it is retarded or inhibited by high concentrations. Arthus & Pagés (1890) demonstrated the importance of calcium for blood coagulation in a few simple experiments. They nevertheless considered that calcium was necessary only for the transformation of fibrinogen to fibrin. They stated: "Le fibrinferment ne peut agir sur le fibrinogène qu'en presence d'un sel calcique." At that time, opinions were at variance with respect to the role played by calcium. Thus, Pekelharing (1891) stated that calcium was necessary for both thrombin formation and fibrin precipitation, whereas Alexander Schmidt postulated that its effect did not differ qualitatively from that of other ions. In 1896, Hammarsten demonstrated incontrovertibly that calcium had no influence on fibrin formation, but acts only in the first phase of blood coagulation.

On the basis of the finding that even his purest fibrinogen preparations, after being converted to fibrin, always left some soluble proteinaceous substances in the supernatant, Hammarsten (1876—1880) suggested that there might be a hydrolytic cleavage of the fibrinogen during coagulation. Hammarsten's attitude to this problem is a consequence of his previous work on casein, in which he had demonstrated a cleavage of the protein in the presence of rennin:

"Die grosse Übereinstimmung, welche zwischen der Gerinnung des Caseins und des Fibrinogens obwaltet, musste noch mehr auffallend erscheinen, nachdem ich gefunden hatte, dass es auch bei der Faserstoffgerinnung nur um zwei Stoffe, einen Eiweisskörper und ein Ferment, sich handelt. Nachdem ich nun früher gefunden hatte, dass bei der Gerinnung einer reinen Caseinlösung mit Lab ein schwerlöslicher Eiweisskörper, der Käse, welcher die unverhältnissmässig grösste Hauptmasse bildet, sich ausscheidet, während ein überaus leichtlöslicher, in äusserst geringer Menge vorhandener Eiweisskörper in den Molken zurückbleibt, lag die Annahme nahe, dass es auch bei der Faserstoffgerinnung um die Spaltung eines Eiweisskörpers, des Fibrinogens, sich handele. In der That können auch die von mir bisher gemachten Beobachtungen kaum in einer anderen Weise gedeutet werden; wenigstens habe ich in allen, bisher ausgeführten Versuchen nach beendigter Gerinnung in dem Serum einen, in sehr geringer Menge vorhandenen, leichtlöslichen Eiweisskörper gefunden, welcher weder Fibrin noch Fibrinogen noch ein aus der Fermentlösung stammender Eiweisskörper ist. Indessen ist das Auftreten eines derartigen Eiweisskörpers in Folge einer bei der Faserstoffgerinnung stattfindenden Spaltung des Fibrinogens noch nicht unzweifelhaft bewiesen, und die hierher gehörenden Untersuchungen sind überhaupt mit so grossen Schwierigkeiten verbunden, dass wahrscheinlich noch geraume Zeit vergehen wird, bevor ich die Resultate dieser Untersuchungen der Öffentlichkeit überzuliefern wage."

The quantity of "fibrinoglobulins" nevertheless varied appreciably in different fibrinogen preparations. Consequently, Hammarsten refrained in later years from taking any definite stand on the matter, since the fibrinoglobulins might have consisted of soluble fibrin, or could possibly have derived from contaminants in the fibrinogen preparations.

Although Huiskamp (1905) had presented evidence in favour of fibrinoglobulins being impurities in the fibrinogen preparations, the concept of splitting of the fibrinogen during its coagulation was developed further by Mellanby (1909). He suggested that fibrinogen is composed of two groups of proteins, and that when coagulation occurs, the bond joining these groups is broken. One group comes out of solution as fibrin, whereas the other remains as a serum globulin. Mellanby saw no grounds for refuting this hypothesis 25 years later, and presumably would have had still less reason to do so today.

Waldschmidt-Leitz, Stadler & Steigerwaldt (1928) suggested that thrombin could be a "trypsin-kinase"-like enzyme, since natural and synthetic trypsin substrates produced inhibition of the coagulation of whole blood. This view was shared by Fuchs & Zakrzewski (1934) who showed that, during the conversion of fibrinogen to fibrin, an increase in non-protein nitrogen occurred in the coagulation mixture, an increase which, in fact, was ascertainable even before visible coagulation. Presnell (1938) observed that the total nitrogen in fibrin formed by the action of thrombin was less than that in a corresponding amount of heat-coagulated fibrinogen. By formol titration, this author also found an increase in the number of free amino groups during the coagulation of fibrinogen.

In 1909 Mellanby had observed that certain snake venoms could replace thrombokinase and calcium in the prothrombin conversion. In 1916, it was incidentally found by Douglas & Colebrook that trypsin had an accelerating effect on blood coagulation. The investigations of Eagle (1937) and Eagle & Harris (1937) showed that different proteolytic enzymes had blood-coagulating activity. Thus, trypsin could transform prothrombin to thrombin, whereas papain induced a fibrinogen-fibrin transformation. Of several snake venom enzymes investigated, some were shown to bring about fibrinogen-fibrin transformation and others prothrombin-thrombin conversion. Based on these observations, the authors suggested proteolysis to be of importance in normal blood coagulation as well.

In 1945, it was suggested by Laki & Mommaerts that a "two-step" reaction was involved in the transformation of fibrinogen to

fibrin in the presence of thrombin. They found that, although no coagulation occurred at slightly acidic pH values, the fibrinogen was changed in some way by the action of thrombin, since the coagulation time after neutralization was shorter for a fibrinogen solution that had been preincubated with thrombin. Laki (1951) suggested that thrombin prepares the fibrinogen molecule in some way for the polymerization which occurs only when the ionic milieu is suitable. These changes in the fibrinogen at low pH values were also effected by thrombic enzymes from snake venoms (Janszky 1949). A small difference in the electrophoretic mobility of fibrinogen and fibrin in urea solution was, in fact, also observed by Mihalyi (1950).

It is evident from this survey that the hypothesis of splitting and unmasking of the fibrinogen in the course of transformation has been conceived and cherished ever since the enzymatic nature of thrombin was established by Alexander Schmidt. This review nevertheless uncovers only one trail in the investigations of the transformation of fibrinogen into fibrin. Other views have perhaps been still more in evidence and, until very recently, even predominant. Thus, as mentioned earlier, Huiskamp considered splitting to be unlikely, since his pure fibrinogen seemed to be converted quantitatively to fibrin. The investigations of Jaques (1938) gave additional support to this view. Although Jaques found that not all the fibrinogen nitrogen could be recovered as fibrin nitrogen, he explained this as a result of the solubility of fibrin. Furthermore, Nanninga (1946), in contrast to Presnell, was not able by means of formol titration to note an increase in free carboxyl groups during fibrinogen transformation. Wöhlisch (*cf.* Wöhlisch 1952) also stated it to be improbable that fibrinogen is split by thrombin, chiefly because it was possible to obtain fibrinogen preparations that are almost completely coagulable. According to Wöhlisch, thrombin has a "denaturase" activity only.

Certain objections can be raised to the earlier investigations made with the object of demonstrating the proteolytic activity of thrombin. The chief criticism concerns the relatively impure fibrinogen and thrombin preparations frequently used. Moreover, the systems were often spontaneously fibrinolytic, which naturally contributed to obscuring the picture. However, it was chiefly the lack of suitable analytic techniques which hampered research along this line.

Direct evidence of a limited proteolytic activity of thrombin was not given until 1951, when Bailey, Bettelheim, Lorand & Middlebrook, by means of Sanger's fluorodinitrobenzene method,

showed a difference between the N-terminal amino acids of bovine fibrinogen and fibrin. Thus, the N-terminal glutamyl residues in the fibrinogen were replaced in fibrin by N-terminal glycyl residues. This was a definite indication that a splitting off of peptide material occurred during the transformation. Furthermore, the earlier indications of a release of non-protein nitrogen during coagulation were confirmed (Lorand 1951, 1952, Kowarzyk 1952). It was later shown by Laskowski, Donnelly, van Tijn & Scheraga (1956) that the release occurred even under conditions where no polymerization took place. In conformity with the indications provided by the studies of Laki & Mommaerts, evidence was also obtained that the proteolytic action of thrombin was fundamentally the same at low pH values, where coagulation was inhibited, as at high pH values where coagulation occurred (Bailey & Bettelheim 1955).

Further studies on the transformation mechanism have been carried out since 1951 with Sanger's technique (Bettelheim & Bailey 1952, Lorand & Middlebrook 1952, 1953, Bailey & Bettelheim 1955). During the transformation of bovine fibrinogen, roughly one N-terminal glutamyl chain disappeared per 330,000 unit weight of fibrinogen. At the same time, two or three glycyl chains appeared in an approximately equal weight of fibrin (Bailey & Bettelheim 1955). Except that the human fibrinogen contained N-terminal alanyl residues instead of the glutamyl residues in bovine fibrinogen, the chemical events during transformation seemed to be of the same nature.

C-terminal residues susceptible to carboxypeptidase are the same in fibrinogen as in fibrin (Bailey & Bettelheim 1955). This indicates that there are possibly no changes in the carboxyl ends of the fibrinogen molecule during coagulation.

From the clot supernatant of bovine fibrin, Lorand (1952) obtained an acidic peptide having glutamic acid as the N-terminal group. The isoelectric point of this peptide was between pH 3 and 4. However, Bettelheim & Bailey (1952) and Bettelheim (1956) isolated by means of electrophoresis two peptides, tentatively named A and B. The A peptide, containing the N-terminal glutamic acid of fibrinogen, had an isoelectric point of about pH 3.1—3.2, thus resembling the peptide obtained by Lorand. The B peptide had a somewhat higher isoelectric point than peptide A. The A and B peptides could also be separated by partition chromatography. Peptide B, in contrast to peptide A, was shown by Bettelheim (1954) to contain tyrosine. The phenolic group of tyrosine was, however, not free but esterified by sulphuric acid. Peptide A

seemed to be released by thrombin at a higher initial rate than peptide B. The C-terminal residues of both peptides have recently been claimed to be arginine (Laki, Gladner & Kominz 1958).

Peptides are also split off from acetylated fibrinogen on incubation with thrombin (Caspary 1956). Fibrinogen acetylated to more than 35—40 % does not form a clot, but partial polymerization can proceed. Peptides are also released from guanidinated fibrinogen when incubated with thrombin, although here the picture seems to be more complex (Kominz & Laki 1954).

The action of thrombin on different synthetic substrates has recently been studied by Sherry & Troll (1954) and Ronwin (1957). Among the substrates tested, the activity towards the trypsin substrate tosylarginine methyl ester was most pronounced. This substrate is also digested by plasmin. In contrast to trypsin and plasmin, thrombin has been claimed to have no activity towards lysine esters (Sherry & Troll 1954). From the claimed C-terminal residues in the peptides and the action of thrombin on synthetic substrates, it might be expected that thrombin would split arginyl linkages in proteins. However, among the proteolytic enzymes, thrombin seems to have a narrow specificity of proteolytic action. Thus, the peptide bonds in ovalbumin and rabbit myosin are not susceptible to thrombin, nor is the glutamyl-arginyl-glycyl-phenylalanine sequence in the B chain of insulin (Bailey & Bettelheim 1955). However, the results of Guest & Ware (1950) suggest that, in high concentrations, highly purified thrombin can further proteolyze fibrinogen and fibrin. Quite recently, the splitting of α casein and β lactoglobulin by thrombin has been reported (Pantlitschko & Gründig 1957). Furthermore, there is by now evidence that gelatin is also split by this enzyme (Miller 1958).

In this connexion, it may be mentioned that Ehrenpreis & Scheraga (1957) found commercial thrombin preparations to be contaminated with another enzyme, probably not identical with plasmin. This enzyme was able to inactivate fibrinogen and the fibrin monomer. At the concentration of thrombin employed, it was not ascertainable in Seeger's highly purified thrombin.

The physico-chemical aspects of the polymerization of fibrinogen, or rather the fibrin monomer, have been intensely studied. The first thorough investigations on the coagulating activity of thrombin under varying conditions were made by Astrup (1944) who studied *e. g.* the effect of ionic strength and pH, and by Ferry & Morrison (1947). The latter authors showed, in a wide range of studies, how the rate of formation of fibrin and the properties of

the fibrin clot are profoundly influenced by pH and ionic strength, and by different reagents, *e.g.* glycerol. They found that the fibrin clots formed under different experimental conditions could be classified as ranging in properties between two extreme types: the *fine* clot, which is transparent, elastic, friable and non-synerizing, and the *coarse* clot, which is opaque, plastic, non-friable, and synerizes very readily. A coarse type is formed at low pH values and low ionic strength. The formation of the fine clot is favoured by increasing pH, increasing ionic strength and increasing temperature. These investigations form the basis of quantitative determination of fibrinogen in different fluids (*cf.* Jacobsson 1955).

Further careful investigations on the influence of a variety of conditions and compounds on the fibrinogen-fibrin transformation have later been carried out by several authors, such as Mihalyi (1948), Ferry & Shulman (1949), Shulman & Ferry (1950) and Edsall & Lever (1951).

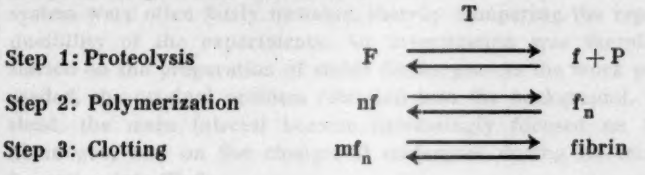
It was shown by Ferry & Shulman (1949) that coagulation of fibrinogen by thrombin could be prevented by the presence of *e. g.* hexamethylene glycol. Although no clot formed, partial polymerization occurred under certain conditions, since the ultracentrifugation diagrams showed two components, one having the same sedimentation constant as fibrinogen, whereas the other was a much heavier component. Its concentration increased with the time of reaction (Shulman & Ferry 1951). The intermediary polymer also appeared in fibrinogen-thrombin systems which had been inhibited by urea (Ehrlich, Shulman & Ferry 1952). Intermediary polymers appeared before coagulation not only in the inhibited systems, but also in uninhibited fibrinogen-thrombin systems (Backus, Laskowski, Scheraga & Nims 1952, Shulman, Ferry & Tinoco 1953, Tinoco & Ferry 1954).

Light-scattering studies on hexamethylene glycol-inhibited fibrinogen-thrombin systems indicated an appreciable formation of an intermediary polymer with a weight-average degree of polymerization of 15, a length of 3500 Å, and a width double that of fibrinogen. The polymer was found to dissociate with dilution (Ferry, Shulman, Gutfreund & Katz 1952). In a study of the changes in light-scattering in a fibrinogen-thrombin system by Steiner & Laki (1951) it was found that, during coagulation of fibrinogen, increasingly large particles were built up through side-by-side and end-to-end association of the apparently unchanged fibrinogen particles. The relative extent of the two kinds of association was found to depend on pH and ionic strength. The changes brought

about by papain were found to be the same as with thrombin.

By means of sedimentation (Tinoco & Ferry 1954) and light-scattering measurements (Casassa 1955) formation of intermediary polymers has also been observed at high pH values in various fibrinogen-thrombin systems.

On the basis of accumulated facts, the following reaction, according to the scheme of Scheraga & Laskowski (1957), may represent the present concept of the transformation of fibrinogen to fibrin in the presence of thrombin:



In this reaction, f denotes the fibrin monomer, and f_n the intermediary polymer. According to these authors, n is a variable number, whereas m is some large number and P represents the fibrinopeptides. Each of these steps is believed to be reversible (Laskowski, Rakowitz & Scheraga 1952).

Inhibition of Thrombin Activity

It was shown by Howell & Holt as early as 1918 that heparin, together with a co-factor in plasma, was a potent inhibitor of the coagulation of fibrinogen by thrombin. Following the discovery by Jorpes in 1935—1936 that heparin is a polysulphuric acid ester of mucoitin, it was suggested that the inhibition may depend on a combination of this strongly negatively charged compound with enzyme and substrate (*cf.* Jorpes 1946). The interaction of heparin with various proteins has, in fact, been demonstrated (Jaques 1943, Nilsson & Wenckert 1954, Gorter & Nanninga 1953).

The coagulating, proteolytic and esterase activities of thrombin are inhibited by diisopropylfluorophosphate (DFP) (Bailey, Bettelheim 1955, Miller & van Vunakis 1956, Gladner & Laki 1956). Thus, thrombin resembles trypsin in its inhibition by DFP and its activity towards tosylarginine methyl ester (TAME).

According to Seegers, Johnson & Fell (1954) physiological inactivation of the coagulating activity of thrombin can follow different routes:

1. Adsorption of thrombin on the fibrin clot.
2. In the presence of heparin and heparin co-factor, the effect of thrombin on fibrinogen is abolished.
3. In plasma and serum, an antithrombin is present which can neutralize large quantities of thrombin. This so-called "progressive antithrombin" does not require the presence of heparin for its action.

Present Investigation

In some studies on the heparin co-factor in 1953, it was observed that the fibrinogen preparations then used in the test system were often fairly unstable, thereby hampering the reproducibility of the experiments. An investigation was therefore started on the preparation of stable fibrinogen. As the work proceeded, the original problem retreated into the background. Instead, the main interest became increasingly focused on the fibrinogen, and on the changes it undergoes during its transformation into fibrin.

Purification of Fibrinogen

In Paper I, a method for purification of fibrinogen in Cohn's fraction I is described. Ethanol fractionation in the cold was adopted. The method is equally well applicable to human as to bovine plasma. Since proteins are, as a rule, more stable in the solid state, and dissolution of fibrinogen in the presence of prothrombin must involve undue risks, we tried to purify fraction I as far as possible by extracting the contaminants. Moreover, extraction is more easily performed under sterile conditions than is fractional precipitation. The advantages of the extraction procedure have been pointed out by Cohn *et al.* (1950).

Dissociation of the protein complexes in fraction I with simultaneous quantitative separation of the components is, however, difficult to achieve by simple extraction with buffer solutions of varying pH and ionic strength. Preliminary experiments nevertheless showed that separation was highly effective when extraction was performed in the presence of glycine. Glycine in high concentration enhances the salting-out effect on the fibrinogen, whereas the salting-in effect on the contaminating proteins is considerable (Fig. 1).

The low solubility of fibrinogen at high concentrations of glycine was observed by Edsall & Lever (1951). In Cohn's method 10, separation of the γ-globulins and the β_1-lipoproteins is facilitated by the presence of glycine.

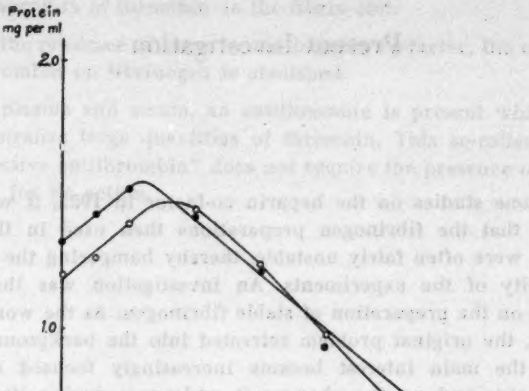


Fig. 1. The effect of glycine on the solubility of bovine fibrinogen.

Method

Fractional extraction. — An investigation on the most suitable conditions for removal of impurities from human and bovine fraction I, leaving the bulk of the fibrinogen in the residue, showed that this could conveniently be performed by extraction with buffers containing glycine under the following conditions: 0.055 *M* citrate buffer, pH 6—7, 1 *M* glycine, 6.5 vol. % ethanol and — 3° C. Under these conditions, 95 % of the fibrinogen was recovered in the residue (fraction I—0), whereas the major part of the impurities were extracted. With this procedure, the coagulability of the protein increased from 40 to 50 % in fraction I to 85 to 90 % in fraction I—0.

Fractional precipitation. — Since no further purification could be achieved by means of extraction, a study was made of the fractional precipitation of the proteins in fraction I—0. This resulted in a procedure in which from a 0.57 % solution of fibrinogen in 0.055 *M* citrate buffer, pH 6.3—6.4, containing glycine to a concentration of 0.12 *M*, a cold-insoluble globulin complex (fraction

I—1) was removed by adding ethanol to 2 vol. % at 0° C. The fibrinogen, precipitated from the supernatant by increasing the ethanol concentration to 6.5 vol. %, was coagulable to 94—97 %, and represented a stable fibrinogen preparation (fraction I—2). Refractionation under the conditions just described resulted in no further increase in purity. Nor did variations in the glycine concentration produce any demonstrable improvement.

Cohn, McMeekin & Blanchard (1938) pointed out that the effect of dipolar ions in solution on the solubility of cystine is influenced by other ions present, and that the effect decreases with rising concentration of the latter. Since the earlier purification steps were carried out at relatively high ionic strength (0.3), it was considered of interest to investigate whether fractionation at lower ionic strength (about 0.1) in the presence of glycine would enhance the purity of the product from fraction I—2. It then proved possible, at a low ethanol concentration, to remove a further cold-insoluble protein complex (I—3). Removal of this complex raised the coagulability of the subsequent fibrinogen fraction (I—4) to between 98 and 100 %.

The different steps in the purification procedure are outlined in Figure 2.

It is to be noted that, in the final fractionation step, different procedures were used for bovine and for human material. In human material, fraction I—3 was precipitated at pH 6.3—6.4. In bovine material, on the other hand, optimal precipitation of I—3 was also obtained at pH 7.0, at which pH the yield of fibrinogen in fraction I—4 was high, i. e., 80—90 % of that in fraction I—2. The yield of human fibrinogen in fraction I—4 was less satisfactory, i. e., about 60 % of the fibrinogen in fraction I—2. The coagulability of the final purified fibrinogen was 98—100 % for both human and bovine material.

Since the publication of Paper I, some 40 bovine preparations have been made. In these preparations, the coagulability of fraction I—2 ranged from 93 to 96 %. The coagulability of fraction I—4 varied between 97 % and in excess of 99 %.

The method described in Paper I has also been used in the preparation of fibrinogen from sheep, goat, rabbit, pig, dog and horse plasma (Paper IV). The coagulability of the purified fibrinogen was between 90 and 98 %, depending on the species.

Protein determination. — The fibrinogen, fibrin and total protein content of fractions coagulable to more than 50 % was determined spectrophotometrically, after dissolving the protein in alkaline urea solution. The method has been described in Papers I

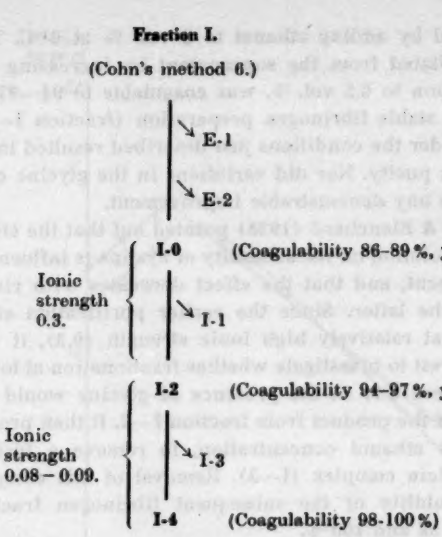


Fig. 2. Scheme of Fractionation.

and II. The extinction coefficient, $E^{1\,\%}_{1\,cm}$, for fibrinogen in alkaline urea is 16.51 at 282 mμ. The value for fibrin is about 2 % higher. Since peptide material is split off from fibrinogen during its conversion to fibrin, and these peptides have very low ultraviolet absorption in the tyrosine-tryphophan region, it is probable that all the phenolic tyrosine and the tryptophan of the fibrinogen remain in the fibrin. Consequently, a coagulability value of 100 % is plausible for pure fibrinogen when a spectrophotometric method of assay is used, and the extinction coefficient of fibrinogen is used for the coagulated sample as well.

Occurrence of Various Other Proteins in the Fractions

Prothrombin. — About 95 % of the prothrombin activity in fraction I was removed by the extractions. During the following steps, the prothrombin activity in the fibrinogen fractions was reduced to values lower than the sensitivity of the test method.

Fibrinolytic enzymes. — Plasminogen and the assumed proactivator of this enzyme were also removed to an appreciable percentage by the glycine extractions. Considerable activity was also found in the supernatants left after precipitating the fibrinogen in the two fractional precipitation steps. No complete removal of

these activities from the fibrinogen has hitherto been achieved, even when the fibrinogen was reprecipitated up to six times. However, plasmin, the active fibrinolytic enzyme, was not ascertainable in the fibrinogen fractions after the extractions.

Antihemophilic globulin (AHG). — The antihemophilic activity of plasma is recovered with good yield in fraction I. This usually applied to fraction I—0 as well. However, on further purification of the fibrinogen, the activity disappeared and could not be recovered in any of the sub-fractions. The final purified fibrinogen possessed inappreciable antihemophilic activity.

Properdin. — It has recently been reported by Rothstein & Pennell (1958) and Sanders, Spicer & Smith (1958) that a considerable amount of the properdin of human plasma is precipitated in fraction I. The properdin could be separated from the fibrinogen in fraction I under the same conditions as used in the present investigation for extraction of fraction I. Starting from this supernatant, the aforementioned authors further purified properdin.

Heparin co-factor. — The heparin co-factor activity of fraction I was fairly low. The remaining activity seemed to be removed by the extraction procedure.

Cold-insoluble globulin complexes. — Cold-insoluble globulin complexes are present in Cohn's fraction I. In the ether method of Kekwick as well, these globulins are precipitated in the fibrinogen fraction, and are subsequently separated from the fibrinogen. The cold-insoluble globulin complexes have been studied previously by Morrison, Edsall & Miller (1948) and by Edsall, Gilbert & Scheraga (1955). Reasons were put forward by the latter authors showing that the cold-insoluble proteins form a complex with fibrinogen.

We consistently found cold-insoluble proteins in fraction I from human as well as from bovine plasma (Paper I). These proteins were concentrated in the two sub-fractions I—1 and I—3, separated at high and low ionic strength, respectively.

In human material, fraction I—1 consisted to about two-thirds of fibrinogen and to one-third of non-coagulable globulins. The recovery of this non-coagulating component from human plasma was found to range from 0.1 to 0.2 g per litre, and from bovine plasma from 0.1 to 0.3 g per litre.

The non-coagulating globulins seemed to be highly susceptible to denaturation. A preliminary N-terminal analysis showed the presence of several N-terminal amino acids, which suggests that the fraction is heterogeneous (Paper II).

Clinical Use of Fibrinogen

In Sweden, fibrinogen has been prepared on a large scale from outdated blood, in principle according to the glycine method, by Kabi AB. It has been used with good results in the treatment of clinical disorders, such as fibrinolysis mainly due to complications in obstetrics and surgery, *e. g.* in connexion with surgery using extracorporeal circulation with a heart-lung machine.

Fraction I—0 has been used in the treatment of hemophilia A and other conditions with AHG deficiency (Blombäck & Nilsson 1958, Nilsson *et al.* 1957).

Properties of Fibrinogen and Fibrin

Electrophoresis. — On free electrophoresis at pH 6.6 and 8.2, the purest fibrinogen migrated as an essentially homogeneous protein during a 10 hours' run. However, after more prolonged electrophoresis at pH 8.2, disturbances in the pattern were observed; the coagulability was simultaneously decreased, indicating that the inhomogeneity was due to partial destruction (Paper I).

Solubility of fibrinogen. — The purified fibrinogen coagulable with thrombin in excess of 99 % is a typical euglobulin. It could easily be redissolved after lyophilization from salt-containing solutions. However, the coagulability decreased by a few per cent on lyophilization (Paper II).

Molecular weight. — By means of light-scattering measurements, the molecular weight of fibrinogen from ox, sheep and goat plasma was estimated to be about 350,000 (Paper III). The molecular weight of horse fibrinogen was found to be higher, i. e., about 500,000. The dissymmetry values for the species investigated are in good agreement with those previously reported for bovine fibrinogen by Katz *et al.* (1952). A length of 500—600 Å was calculated for all species, on the assumption of the molecule being a stiff rod.

Nitrogen. — The nitrogen content of bovine fibrinogen and fibrin was found to be 16.7 and 17.0 %, respectively (Paper II).

Carbohydrate components. — Several carbohydrate components were identified in purified fibrinogen and fibrin (Paper II). Thus, galactose and mannose could be identified by paper chromatography. Small amounts of sialic acid and glucosamine were also present.

Whereas the figures for hexosamine as well as for sialic acid are

the same in fibrinogen and fibrin, the amount of hexoses seems to be somewhat less in the latter than in the former, and may indicate a release of carbohydrates during transformation, as suggested by Szára & Bagdy (1953) and Bagdy & Szára (1955). It must, however, be pointed out that the error of the method in the orcinol reaction is considerable.

The sialic acid in bovine fibrinogen and fibrin is probably of the ovine type, despite the fact that only the sialic acid II of Yamashina (1956) could be identified. Yamashina has pointed out that, in the α_1-acid glycoprotein, the sialic acid II is probably a derivative of the ovine type of sialic acid. The longer the acidic hydrolysis for liberation of sialic acid, the more of the ovine type of sialic acid is converted to this derivative. This difference between the sialic acid in fibrinogen and α_1-acid glycoprotein may indicate differences in the binding of the sialic acid in the two proteins. Obviously, the possibility also exists that the sialic acid initially present in fibrinogen is of an entirely different type to that in α_1-acid glycoprotein.

The carbohydrate components seem to be well anchored in the fibrinogen and fibrin structure, in view of the fact that repeated refractionation at low alcohol concentrations did not change the amount.

N-terminal Amino Acids in Fibrinogen and Fibrin

Since N-terminal analysis had been made on fibrinogen only with Sanger's fluorodinitrobenzene method, it was found of interest to confirm the results on our purest fibrinogen with the use of Edman's phenylthiocarbamyl method. Edman's method offers several advantages over the method of Sanger. The cleavage and cyclization of the phenylthiocarbamyl (PTC) amino acid residues can be performed under comparatively mild conditions, and destruction during the procedure is small.

In the paper-chromatographic systems described by Edman and Sjöquist, the resulting phenylthiohydantoins (PTH) of the N-terminal amino acids can easily be identified and estimated quantitatively (Sjöquist 1953, Edman & Sjöquist 1956, Sjöquist 1957, Sjöquist 1958).

A drawback inherent in Sanger's technique for N-terminal analysis is the destruction of dinitrophenyl (DNP) amino acids, the extent of which is somewhat difficult to predict, during hydrolysis of DNP proteins. The destruction numbers obtained by de-

termining the yield of free DNP amino acids after heating in 6 *N* HCl, with or without the presence of protein, are not always applicable to the N-terminal residues in an unknown protein, as has been pointed out by many workers. As far as fibrinogen is concerned, the difference in the number of terminal residues obtained by various authors with the DNP method may be ascribed to the different destruction number used (Bailey & Bettelheim 1955).

In Edman's method as applied by us, the estimation of the overall yield was carried out on free amino acids in the presence of a certain amount of protein. This was because, in the absence of protein, some of the PTC and PTH amino acids are appreciably destroyed by the side-reaction products formed during the determination procedure, under the conditions used by us, i. e., 1 *N* HCl at 100° C. The yields thus obtained could be used for correction of the experimental values for the different N-terminal amino acids. The loss of PTH amino acids could be ascribed mainly to the loss in paper chromatography, indicating that destruction of PTH amino acids occurring during determinations on protein is small.

The techniques used in the present investigation on the N-terminal amino acids in highly purified fibrinogen and fibrin have been described in Paper IV (Blombäck & Yamashina 1958).

The observations on N-terminal amino acids in bovine fibrinogen and fibrin made by Bailey, Bettelheim, Lorand & Middlebrook, applying Sanger's method, have been confirmed in all essentials. Differences in quantity were, however, present.

Table 1

N-terminal amino acids in bovine fibrinogen and fibrin
(Fg = fibrinogen; F = fibrin)

The N-terminal amino acids are expressed in moles per 340,000 g of protein. The figures within brackets denote the most probable number in this unit.

	Lorand & Middlebrook (1952) DNP method			Bailey & Bettelheim (1955) DNP method			Present authors (1958) PTC method		
Protein	Tyr.	Glu.acid	Gly.	Tyr.	Glu.acid	Gly.	Tyr.	Glu.acid	Gly.
Fg	1.5	0.7	—	2.3	1.3	—	1.9	1.7	—
	(2)	(1)		(2)	(1)		(2)	(2)	
F	1.4-1.6		3.0-3.7	2.3	—	2.7	1.9	—	3.9
	(2)		(4)	(2)		(2—3)	(2)		(4)

The results obtained by different workers are summarized in Table 1. It can be seen that our values for the tyrosyl residues agree with those obtained by Lorand & Middlebrook (1952) and by Bailey & Bettelheim (1955). There is, however, a difference as to the number of glycyl and glutamyl residues. Moreover, it could be shown that, in bovine fibrinogen, at least part of the γ-carboxylic groups in the N-terminal glutamic acid are in the amide form. The results show that fibrinogen and fibrin are different entities from a chemical point of view as well.

In addition, the investigations on the N-terminal amino acids were extended to fibrinogen and fibrin from different species. With the methods used for purification of fibrinogen, it was possible to obtain fibrinogen preparations of about the same high degree of purity from the different species. No spots which could be ascribed to protein impurities were seen on paper chromatograms in the N-terminal determination. In the scheme in Fig. 3, the most probable number of polypeptide chains in the molecule from different species has been suggested. The number of residues calculated are the most probable in a 350,000 unit weight of human, bovine, pig, sheep, goat and dog fibrinogen, and in a 500,000 unit weight of horse fibrinogen. The circles indicate chains with no free N-terminal groups, possibly cyclic chains, since no other amino acids have hitherto been found. However, because of difficulties in analyzing N-terminal cysteine, arginine and histidine in proteins with the technique used by us, the possibility cannot be ruled out that cysteinyl, arginyl or histidinyl residues might be present in some fibrinogens, instead of the indicated cyclic ones. The solution of this problem must await the isolation and analysis of the different peptides split off.

It is evident from the present results that the tyrosyl residue, whatever its function in the polymerization process may be, is a feature common to fibrinogen and fibrin from all species investigated. The amount of N-terminal tyrosine on a weight basis of fibrinogen and fibrin is about the same in all species, except in the horse. In fibrin, only glycine and tyrosine were found as N-terminals.

In addition to tyrosine, at least one other N-terminal amino acid is always present in fibrinogen. In bovine fibrinogen, it consists of glutamic acid, in horse and dog of threonine, and in human, pig, sheep and goat of alanine. In sheep and goat fibrinogen, glycine is present as a third N-terminal amino acid. Since the pattern of the terminal amino acids varies, the additional ones do not seem to be included in the specific requirements for

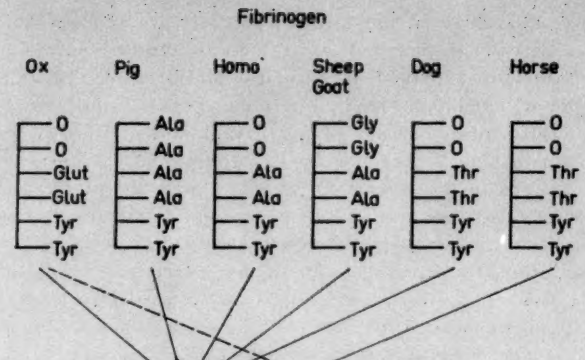


Fig. 3. N-terminal amino acids in fibrinogen and fibrin from different species.

the transformation of fibrinogen to fibrin by the thrombic enzyme.

There is a difference between the N-terminal amino acid composition of fibrins obtained from plasma and those from purified fibrinogen solutions (Paper II). Thus, a small amount of N-terminal aspartic acid in addition to tyrosine and glycine was found in clots from plasma, indicating occlusion of some plasma proteins within the clot. It is suggested that the occluded protein may consist of albumin. The relation between tyrosine and glycine was the same in the plasma clot as in the other type of clot.

Transformation of Fibrinogen to Fibrin

Isolation of fibrinopeptides. — As suggested from the results presented in Paper IV, different kinds of peptides seem to be split off from the fibrinogen of the various species. In bovine fibrinogen, two glutamyl and two cyclic peptides are likely to be released by the action of thrombin. For further analysis of the transformation

process, a study of these peptides seemed desirable, especially since opinions were somewhat at variance both with regard to composition as well as number of peptides (*cf.* Bettelheim 1954, Lorand 1954, Bettelheim 1956).

Peptides from a tryptic hydrolysate of ribonuclease had been successfully separated by chromatography on cation exchangers of low cross-linking (Hirs, Moore & Stein 1956). It fairly soon became evident that this type of ion exchanger could also conveniently be used for the chromatographic separation of peptides released during the coagulation of bovine fibrinogen. The chromatography of bovine fibrinopeptides on 2 % cross-linked Dowex-50, on both a large and small scale, has been described in Paper V (Blombäck & Vestermark 1958). The peptides in the clot supernatant were adsorbed at pH 3.1—3.2 on the ion exchanger preequilibrated with 0.1 *M* formate buffer. The step-wise elution of the fibrinopeptides was accomplished with 0.1 *M* acetate buffers at pH values betwen 4.3 and 5.0. Three peptides usually appeared, E, A and B in the order mentioned. Of these peptides, only peptides A and B seem to be relevant to the problem of fibrinogen transformation. Although peptide E was always observed in the chromatograms from the clot supernatant, the amount of this component was small. It might have been a contaminant of the fibrinogen preparation, or perhaps a denaturation product of the peptides.

Table 2

Amino acid composition of bovine fibrinopeptides A and B

Amino acids	A peptide		B peptide	
	μ moles per mg peptide	g per 100 g peptide	μ moles per mg peptide	g per 100 g peptide
Alanine	absent		0.371	3.3
Arginine	0.396	6.9	0.632	11.0
Aspartic acid	1.220	16.2	1.340	17.8
Glutamic acid	0.862	12.7	1.110	16.3
Glycine	2.170	16.3	1.070	8.0
Isoleucine / Leucine	0.407	5.3	0.329	4.3
Lysine	absent		0.195	2.8
Phenylalanine	0.398	6.6	0.366	6.0
Proline	0.818	9.4	0.618	7.1
Serine	0.546	5.7	absent	
Threonine	0.311	3.7	absent	
Tyrosine	absent		0.332	6.0
Valine	0.399	4.7	0.399	4.7

In addition to the three peptides mentioned, small amounts of two others were occasionally detected. In two experiments they constituted 5—8 % of the total peptides obtained.

The mean value for the amount of peptide B obtained from four batches of fibrinogen was approximately 9 mg per gram fibrinogen. The mean value for peptide A in the same preparations was approximately 7 mg. The recovery of peptide A showed greater variations than that of peptide B. Both peptides are white, occasionally hygroscopic solids. They are easily soluble in water and aqueous organic solvents, such as aqueous acetone, methanol and ethanol, at high concentration of the organic component.

Both peptides seemed to be essentially devoid of neutral sugars, as judged by the orcinol reaction.

A preliminary amino acid analysis of the A and B peptides is shown in Table 2. The figures are for air-dry substance. The analysis was performed with the method of Sjöquist (1957), except that the PTH amino acids were determined quantitatively by paper chromatography (Sjöquist 1958.)[1] No correction factors for destruction have been applied in the calculations. Qualitatively, the results are in good agreement with those of Bettelheim for fibrinopeptides A and B isolated by means of electrophoresis and reversed phase partition chromatography on Hyflo Super-Cel.

In paper electrophoresis at pH 4.1 in 0.05—0.10 *M* acetate buffers, the A peptide had a somewhat greater acidic net charge than peptide B. Peptide B, in contrast to peptide A, contained tyrosine, the phenolic group of which was, however, not free, but seemed to be esterified by sulphuric acid, as suggested by Bettelheim (1954). This was evidenced by the lack of ultraviolet absorption in the tyrosine region. After hydrolysis of peptide B for 4 minutes in 1 *N* HCl on the boiling water bath, the typical ultraviolet absorption of tyrosine appeared.

After alkaline hydrolysis in baryta water for 24 hours at + 125° C, tyrosine-0-sulphate could be identified by chromatography on Dowex-1 according to Tallan, Bella, Stein & Moore (1955). In most batches of peptide B, all the tyrosine was found to be conjugated. The tyrosine content shown in Table 2 would correspond to 9 % tyrosine-0-sulphuric acid or 1 % sulphur in peptide B. In one peptide batch, a content of only 3 % tyrosine-0-sulphuric acid was found by chromatography. In this particular batch, however, the tyrosine ester had been partly split, probably

[1] The analyses were carried out by Dr. J. Sjöquist, University of Lund, Lund, Sweden.

Table 3
N-terminal glutamic acid in peptide A

Peptide A preparations	Amount of peptide containing one mole glutamic acid g
BP 6	2452 a
BP 6	3620 b
BP 9	2980 b
BP 10	3431 a
BP 11	3010 a

a) Determination by the author. The figures are corrected with respect to the yield of glutamic acid in the determination procedure (Paper IV).

b) Determination by Dr. Sjöquist. No correction was made for yield.

during preparation of the peptide, since an appreciable tyrosine absorption in the ultraviolet was present already before hydrolysis.

Because of shortage of material, no quantitative sulphur determination has yet been made, but a fine precipitate appeared after addition of $BaCl_2$ to the hydrochloric acid hydrolysate of the B peptide.

N-terminal analysis of peptide A showed the presence of glutamic acid. One mole of glutamic acid was found in approximately 3000 g of peptide, both by the author and by Sjöquist, who used another technique with milder conditions for cleavage and cyclization. The results with different preparations are shown in Table 3. It was observed that a considerable destruction of the N-terminal glutamic acid derivatives took place when cleavage and cyclization of the PTC peptide were performed in 1 *N* HCl at 100° C. If, however, the determination was performed in the presence of protein, the losses were minimized. Therefore, the determination of the N-terminal glutamic acid in bovine peptide A was always performed in the presence of purified human fibrinogen, having tyrosine and alanine as N-terminals (Paper V). Except for glutamic acid, no N-terminal amino acid was found, neither PTH amino acids soluble in ethyl acetate, nor in water. Chromatography for identification of water-soluble PTH amino acids, including PTH cysteic acid, was performed in the solvent system described by Sjöquist (1957).

The amino acid sequence of peptide A from the amino end was: glu. asp. gly.[1] for the first three residues.

No N-terminal amino acid could be detected in peptide B.

Oxidation of peptide B with performic acid at 0° C according to Hirs (1956) and subsequent N-terminal determination gave no support for the occurrence of N-terminal cysteine in this peptide.

From the N-terminal analysis it seems likely that the molecular weight of peptide A is about 3000. Approximately the same minimum molecular weight was calculated for peptide B from the amino acid composition (Table 2).

It was suggested in Paper IV that two glutamyl and two cyclic peptides were released during the transformation of bovine fibrinogen to fibrin. Assuming a molecular weight of 340,000 for fibrinogen and 3000 for each of the peptides, roughly 3—4 % of peptide material would then be expected to be released during coagulation of fibrinogen. The peptides recovered from the clot supernatant usually constituted less than 2 % of the fibrinogen If, however, the yields were taken into account, the peptides were calculated to constitute between 3 and 4 % of the fibrinogen. (Table 4). The yield of both peptides was assumed to be the same as the recovery of glutamyl residues in peptide A, expressed in per cent of those originally present in the fibrinogen.

Table 4

Recovery of peptides from clot supernatants of bovine fibrinogen

The peptides were obtained from 1 g of fibrinogen

Prep. No.	Amount of peptides found mg	Yield of glutamyl residues in peptide A in % of those in fibrinogen	Corrected amount of peptides mg
BP 6	11.3	31.3	36.2
BP 11	23.3	73.0	32.0

On the basis of the studies in Papers IV and V and the additional data presented here, it seems justified to suggest that, during the conversion of bovine fibrinogen to fibrin, two moles of peptide A and two of peptide B are released per mole of fibrinogen.

Kinetic aspects of the proteolytic action of thrombin. — In Paper V, chromatographic analysis of the peptides released during the fibrinogen-fibrin transformation gave strong evidence of the

[1] The work on the amino acid sequence of peptide A is in progress and the results will be published elsewhere by Dr. Sjöquist and the author.

conclusion that the release of peptide A and B, respectively, occurred at different initial rates. A difference in the rate of release of the two peptides had also been noticed by Bettelheim (1956). It was found that in the initial phase of transformation, mainly peptide A was released. At a point when about 40 % of the A peptide had been split off, only about 8 % of peptide B could be recovered. In later stages, successively increasing amounts of peptide B appeared. Although no conclusive data were obtained with respect to the relative maximum rate at which the two peptides were split off, the over-all rate for peptide B seemed to be slower. It was furthermore shown that the release of peptide A ran parallel with the fibrin formation (Fig. 4), whereas peptide B was released at a maximum rate when fibrin formation was near completion. This afforded strong evidence of the possible importance of the release of peptide A for subsequent polymerization.

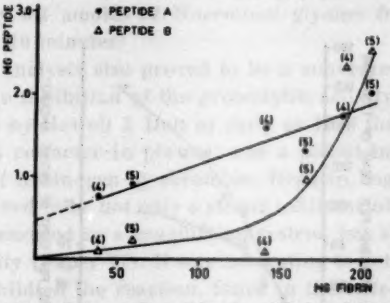


Fig. 4. Release of peptides A and B in relation to fibrin formation. The bracketed figures denote the experiments within each series.

The results obtained by chromatography (Paper V) were confirmed by direct N-terminal determinations in fibrinogen-thrombin systems at different pH values, both when coagulation occurred at pH 6.3, as well as in a system at pH 9.0, where coagulation usually did not take place under the experimental conditions in question (Paper VI). The time displacement in the release of the two peptides during conversion of fibrinogen to fibrin resulted in a diphasic curve when the N-terminal glycyl residues which appear in a fibrinogen-thrombin mixture were plotted against time (Fig. 5). Since peptide A contains glutamic acid as N-terminal residue, the release of peptide A from fibrinogen could also be studied by measuring the rate of decrease in glutamyl residues in fibrinogen during incubation with thrombin. It is evident that,

under different experimental conditions, the glutamyl residues released were equal in number to the glycyl residues that appeared up to a point where about 50—70 % of the glutamyl residues originally present had been split off (Fig. 5). From these experiments, it seems justified to conclude that, during the action of thrombin on fibrinogen, mainly peptide A is released in the initial stage.

Fig. 5. Appearance of N-terminal glycine and release of N-terminal glutamic acid. Thrombin concentration: 0.02 NIH units/ml. △, glu;. ●, gly.

These findings permit a more detailed investigation of the kinetics of the release of peptides A and B during the fibrinogen-fibrin transformation. Some investigations on this line were made (Paper VI). Thus, the dependence of the proteolytic action of thrombin on pH, ionic strength, temperature and enzyme and substrate concentration was measured. Since we were dealing with the initial course of this reaction, it was mainly changes in the release of peptide A that were measured. The pH optimum for the proteolytic action of thrombin on its natural substrate was between 8.0 and 9.0 in tris-imidazol buffer, or the same as for its esterase activity measured on tosylarginine methyl ester in tris buffer (Papers III and VI). Thus, with regard to optimum pH, thrombin resembles trypsin in its action on these substrates. The proteolytic action of thrombin was, in similarity to that of other proteolytic enzymes, also influenced by temperature, ionic strength, and enzyme and substrate concentration (Paper VI). The optimal ionic strength

was about 0.1 and the optimal temperature between 30 and 40° C. *The initial rate of appearance of N-terminal glycine was proportional to the enzyme concentration.* With a given enzyme preparation, there was fairly good reproducibility with respect to the proteolysis on different batches of fibrinogen. Thus, under the conditions described in Paper VI, one NIH unit of thrombin released approximately 0.1 μmoles of N-terminal glycine from 75 mg of fibrinogen in 10 minutes.

N-terminal analysis also proved to be a convenient tool for investigating the inhibition of the proteolytic activity of thrombin. It was shown by Howell & Holt as early as 1918 that heparin, together with a co-factor in plasma, was a potent inhibitor of the coagulation of fibrinogen by thrombin. Heparin, together with its co-factor, proved to be not only a strong inhibitor of the thrombin activity as measured in a coagulating system, but also of its proteolytic activity (Paper VI). It was interesting to note that heparin alone also inhibited the reaction. Since in the latter experiments contamination with co-factor most probably was absent, it seems likely that the inhibiting action of heparin alone depends on a complex formation of heparin, which has a strongly negative electric charge, with charged groups on the substrate as well as on the enzyme, thereby hampering the enzyme substrate combination.

In the experiments described above, bovine thrombin was exclusively used in studies of the fibrinogen-fibrin transformation. It is known that other enzymes can also induce a fibrinogen-fibrin conversion, *e. g.* certain snake-venom enzymes and papain. Reptilase, the coagulating enzyme from the venom of *Bothrops jararaca,* was incidentally found to have very interesting properties in this respect (Paper VII). It was apparent from the experiments that Reptilase had a thrombin-like effect in the second stage of coagulation, even when tested on a highly purified fibrinogen solution free from other coagulation factors. Earlier investigators had also found this venom preparation to have a thrombin effect, but suggested in addition that it promoted the prothrombin conversion. Our experiments did not, however, indicate any such latter effect. Thus, the effect of Reptilase was the same, irrespective of whether prothrombin was present in the plasma. The shortening of the recalcification time of plasma on addition of Reptilase could be explained entirely by the thrombin activity of the preparation. Furthermore, it was shown that Reptilase had no influence at all on the prothrombin consumption of hemophilic plasma or of normal plasma. Contrary to what might have

been expected if it had any factor V activity, Reptilase did not correct the prolonged one-stage prothrombin time of stored plasma.

The resemblance of Reptilase to thrombin was further accentuated by its action on tosylarginine methyl ester. Reptilase had only a slight fibrinolytic effect, if any (Papers VI and VII).

Coagulation studies nevertheless disclosed differences between the properties of thrombin and Reptilase. Thus, Reptilase was not inhibited by heparin plus co-factor, nor by the plasma antithrombin. Furthermore, Reptilase did not seem to be absorbed on the fibrin clot.

A study of the N-terminal amino acids in bovine fibrinogen and in fibrin formed by thrombin and Reptilase, respectively, showed a similarity in the mode of action. Thus, the N-terminal glutamyl residues in fibrinogen disappeared and were replaced by glycyl residues in the fibrin. Quantitatively, however, there was a difference, since the molar ratio of tyrosine to glycine in the thrombin fibrin was 1:2, but in the Reptilase fibrin 1:1 (Fig. 3).

The type of fibrin formed by the action of papain was found to be of essentially the same type as Reptilase fibrin, at any rate with respect to the N-terminal residues (Paper IV).

The earlier indication that only peptide A was split off by Reptilase was confirmed by means of chromatography of the clot supernatant after coagulation of fibrinogen with Reptilase (Paper VI).

From these experiments it can be concluded that, if proteolysis is necessary for the subsequent polymerization, splitting off of peptide A is indispensable for initiating polymerization.

Proteolysis and polymerization. — Although the appearance of N-terminal glycyl residues and the polymerization of fibrinogen are essential features of the thrombin action, the two processes do not follow each other stoichiometrically. Their dependence on pH was studied in Paper III, by light-scattering and N-terminal techniques (Blombäck & Laurent 1958). Each of these reactions was found to be influenced in different ways by changes in pH.

It can be inferred from Fig. 6 that the initial appearance of N-terminal glycine under the action of thrombin is a rectilinear function of time for all pH values. On the other hand, the upper part of the figure shows that this certainly does not apply to the polymerization rate. At high pH values the rate is almost linear, but at lower pH values, *e. g.* 6.35, the speed of polymerization suddenly increases after a lag-phase. If the polymers are built up by adding fibrin monomer molecules one by one to the chain,

either laterally or end-to-end, the rate should be linear. It is, however, also possible that aggregates are added to aggregates, and in this case an exponential increase in molecular weight could be expected, which seems to be more likely, at least at pH 6.4. The cause of this sudden aggregation may also be an oversaturation of the solution with respect to the intermediary product, soluble fibrin, which has been shown to be formed in appreciable quantities by the action of thrombin at this pH value (Papers III and VI).

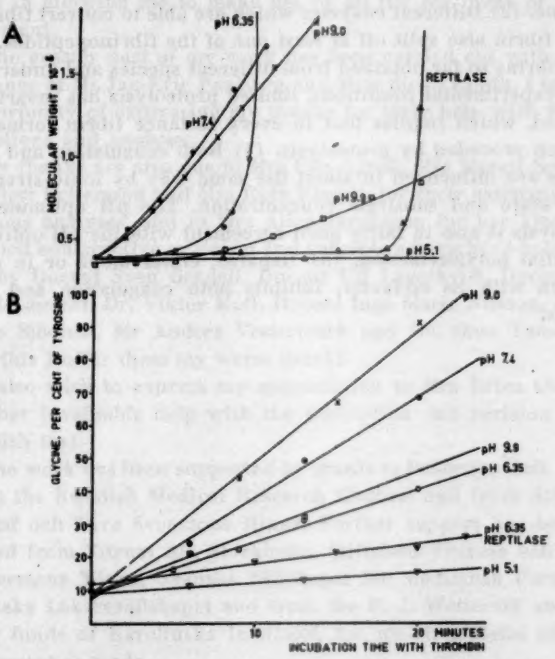


Fig. 6. Polymerization (A) and appearance of N-terminal glycine (B) in a fibrinogen solution after addition of thrombin at different pH values, or of Reptilase at pH 6.35.

It was found that the initial polymerization at lower pH values (6.4 and 7.4) is mainly end-to-end, whereas at higher values (9.0 and 9.9) lateral aggregation also occurs initially.

In contrast to the over-all polymerization process, it was, however, found that the initial polymerization rate showed essentially the same dependence on pH as did proteolysis.

It has repeatedly been suggested that proteolytic splitting off

of a peptide from the fibrinogen molecule might be indispensable for subsequent polymerization. Although direct evidence supporting this hypothesis is still lacking, several facts afford indirect evidence of the importance of the proteolytic reaction occurring at the amino end of the polypeptide chains for the subsequent polymerization. These facts are: (1) It has so far been impossible to separate the coagulating and proteolytic activity of thrombin. Thus, the same changes in the N-terminal amino acid pattern are produced by a highly purified thrombin preparation as by a less pure one. (2) Different enzymes which are able to convert fibrinogen to fibrin also split off at least one of the fibrinopeptides. (3) In all fibrins so far obtained from different species and under different experimental conditions, limited proteolysis has invariably occurred, which implies that in every instance fibrin formation has been preceded by proteolysis. (4) Both coagulation and proteolysis are influenced in about the same way by ionic strength, temperature and substrate concentration. The pH optimum for proteolysis is also in fairly good agreement with the pH optimum for initial polymerization. (5) Heparin, either alone or in combination with its co-factor, inhibits both coagulation and proteolysis.

Acknowledgements

It is a pleasure to acknowledge my great debt of gratitude to my Chief, Professor Erik Jorpes, for his unfailing interest in my work, and for his guidance and encouragement throughout the course of the investigation. I am also deeply grateful for his kindness in allowing me to make use of all the resources of his department.

The greater part of my work has been carried out with the assistance of Mr Jan-Eric Paulsson and Miss Birgit Lindh. I take this opportunity of expressing my thanks for their help with the preparations and analyses.

My thanks are also due to Mrs Ella Agrell, Mrs Margit Sohlberg, Mr Bert Pettersson and Mrs Ruth Flagell, for their assistance with various analyses, and to Mrs Alice Jacobsson for her kind help.

Much constructive criticism and valuable advice have been given me by Docent Sven Gardell, Docent Ulf Lagerkvist, Docent Torvard Laurent, Dr. Viktor Mutt, Docent Inga Marie Nilsson, Docent John Sjöquist, Mr Anders Vestermark and Dr. Ikuo Yamashina. For this I offer them my warm thanks.

I also wish to express my appreciation to Mrs Erica Odelberg for her invaluable help with the translation and revision of the English text.

The work has been supported by grants to Professor Erik Jorpes from the Swedish Medical Research Council and from Stiftelsen Gustaf och Tyra Svenssons Minne. Further support has been obtained from Vitrum AB, Stockholm, Stiftelsen Therese och Johan Anderssons Minne, Svenska Sällskapet för Medicinsk Forskning, Svenska Läkaresällskapet and from the P. J. Wettervik and Ivar Berg funds of Karolinska Institutet, for which grateful acknowledgement is made.

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